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AKT is critically involved in cooperation between obesity and the dietary carcinogen amino-1-methyl-6-phenylimidazo [4,5-*b*] (PhIP) toward colon carcinogenesis in rats

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ABSTRACT

Obesity is highly associated with colon cancer development. Whereas it is generally attributed to pro-tumorigenic effects of high fat diet (HFD), we here show that a common genetic basis for predisposition to obesity and colon cancer might also underlie the close association. Comparison across multiple rat strains revealed that strains prone to colon tumorigenesis initiated by a dietary carcinogen amino-1-methyl-6-phenylimidazo [4,5-*b*] pyridine (PhIP) tended to develop obesity. Through transcriptome and extensive immunoblotting analyses, we identified the basal level of activated AKT in colonic crypts as a biomarker for the common predisposition. Notably, PhIP induced activation of AKT, which could persist for several weeks under a low fat diet (LFD), but not under HFD. On the other hand, PhIP and HFD independently induced Wnt pathway activation and inhibited apoptosis, through distinct mechanisms involving GSK-3β, caspase 3 and poly-ADP ribose polymerase (PARP). Taken together, these observations provide mechanistic insights into how PhIP-induced activation of AKT might cooperate with HFD at multiple levels toward development of colon cancer.

1. Introduction

Colorectal cancer (CRC) is a leading cause of cancer death worldwide [1]. In the multi-step development of sporadic CRC, Wnt pathway activation is the most frequent initiating event, typically achieved by functional loss of adenomatous polyposis coli (APC) or activating mutation of CTNNB1 encoding β-catenin [2]. Subsequent progression to full-blown tumors is mediated by accumulation of genetic alterations in tumor suppressor genes and oncogenes [3], or by environmental factors, including inflammation. In fact, inflammatory bowel disease is a high-risk condition for CRC in humans [4], and dextran sodium sulfate (DSS)-induced colitis accelerates azoxymethane-induced colon tumorigenesis in mice [5]. Obesity-associated visceral fat or adipocytes have recently emerged as a source of inflammation [6]. Leptin and adiponectin, a class of cytokines secreted by adipocytes, are mediators of inflammation by binding to their specific receptors [7]. Genetic ablation of these pathways in mice indeed affected tumorigenicity under a high fat diet (HFD), confirming the pro-tumorigenic nature of obesity [8,9].

Amino-1-methyl-6-phenylimidazo [4,5-*b*] pyridine (PhIP) is a heterocyclic amine (HCA) abundantly contained in cooked meat. It binds to DNA and forms adducts, which could in turn induce mutations, thereby potently inducing tumors in the colon, prostate and mammary glands in rats [10]. Notably, these types of tumors are all closely associated with westernized high-fat diets in humans, and HFD indeed accelerated PhIP-initiated carcinogenesis in these organs in rats [11]. PhIP administration recapitulates multi-step colon tumorigenesis from aberrant crypt foci (ACF), dysplasia, adenoma, and adenocarcinoma [12]. Besides, PhIP-induced tumors frequently harbor mutations in APC and CTNNB1, similar to human CRC [13]. These observations strongly suggested that PhIP might be a major environmental carcinogen for human CRC.

Although ACF are not *bona fide* pre-neoplastic lesions of the colon, susceptibility of strains to chemically-induced tumorigenesis is conveniently estimated by the number of ACF at an early point, largely due to their high correlation, shorter period of time for observation, and higher incidence [14]. The numbers of ACF induced by PhIP vary among inbred strains [12], strongly suggesting that multiple genetic factors determine the susceptibility to colon carcinogenesis. In an effort to identify these loci, we noted that rat strains with more ACF tended to manifest a more severe obese phenotype, which prompted us to investigate the molecular

Abbreviations: PhIP, amino-1-methyl-6-phenylimidazo [4,5-*b*] pyridine; HCA, heterocyclic amine; ACF, aberrant crypt foci; GSEA, gene set enrichment analysis.

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basis underlying the common predisposition. We clarified the relevance of AKT in the colonic crypts in linking obesity to PhIP-induced CRC, providing mechanistic insights into the cooperation between obesity and CRC.

2. Materials and methods

2.1. Rats, diet and chemicals

We purchased BUF, F344 and ACI rats from CLEA Japan (Tokyo, Japan), LEW, WKY and BN from Charles River Japan Inc. (Yokohama, Japan), and WKAH, OM, DA and KND from Japan SLC (Hamamatsu, Japan). PVG, DON, LEA, DRH, WF, SDJ, LE and NIG-III were provided from The National BioResource Project (NBRP) for the Rat (Kyoto University, Kyoto, Japan). Animal studies were carried out according to the Guideline for Animal Experiments, drawn up by the Committee for Ethics in Animal Experimentation of the National Cancer Center, which meet the ethical standards required by the law and the guidelines about experimental animals in Japan. Five-week-old male rats were fed a low fat diet (LFD) AIN-93G (Dyets Inc., Bethlehem, PA) for 1 week. To induce ACF, rats were fed LFD containing 400 ppm of amino-1-methyl-6-phenylimidazo [4,5-*b*] pyridine (PhIP) (Nard Institute, Osaka, Japan) for the first 2 weeks, followed by a high fat diet (HFD) containing hydrogenated oil PRIMEX (Dyets) for 4 weeks. To induce tumors, this cycle was repeated three times, and experimental animals were fed a HFD for the rest of the course of experiments, to conduct an intermittent PhIP feeding protocol [11]. N-acetoxy-PhIP (Nard Institute), an active form of PhIP, was used for an *in vitro* experiment.

2.2. Evaluation of obesity and tumorigenicity

After fasting for 16 h, serum and body fat were collected on sacrifice at 12 weeks of age. All the blood biochemistry data were obtained by SRL Inc. (Tokyo, Japan). Body weight and body fat weight were measured at 8, 10, and 12 weeks of age. Visceral fat was harvested from epididymal, mesenteric, perirenal and retroperitoneal fat pads. Subcutaneous fat was collected from the dorsal skin. Total body fat weight was calculated as the sum of visceral and subcutaneous fats. The colons were fixed by 10% neutralized formalin overnight and stained with 0.2% methylene blue for 15 min to count the numbers of ACF, aberrant crypts (ACs), and tumors under a stereoscope. Paraffin-embedded thin sections at 5 μm were subject to hematoxylin and eosin staining for histological analysis.

2.3. Colon crypt isolation

Colonic fragments of 1–2 cm long were washed several times with TBS, and subject to incubation at 37 °C for 30 min in Hanks' balanced salt solution supplemented with 30 mM EDTA, 5 mM PMSF, 40 mM NaF and 5 mM sodium pyrophosphate decahydrate. Isolated crypts were stored at −80 °C until used for further analysis. RNA was extracted with TRIzol reagent (Invitrogen, Tokyo, Japan). Protein was extracted with T-PER Tissue Protein Extraction Reagent (Pierce, Alabama) supplemented with Complete Mini (Roche Diagnostics, Mannheim, Germany) and Halt Phosphatase Inhibitor (Pierce).

2.4. Cell culture

Normal human colon cells FHC were cultured in media containing 10% FBS and supplemented with penicillin and streptomycin. 1 day prior to experiments, the culture supernatant was replaced with serum-free media. N-acetoxy-PhIP, an activated form of PhIP, was dissolved in DMSO and added to the cells at 10 μM.

2.5. Microarray analysis

Labeled cDNA synthesized from 500 ng of total RNA was hybridized with Agilent Whole Rat Genome 4x44K microarrays, G4131F (Agilent Technologies), following the manufacturer's instructions. Hybridization images were scanned by High Resolution Microarray Scanner (Agilent Technologies), and analyzed with Agilent Feature Extraction Software v9.5. Raw data were analyzed by Gene Spring GX 7.3.1. Gene set enrichment analysis (GSEA) was conducted with GSEA software [15].

2.6. Western blotting

The proteins were separated by SDS–PAGE and transferred to PVDF membranes. The primary antibodies against p-AKT (Ser473), AKT, FOXO1, FOXO3a, FOXO4, Bim, Caspase-3, p-GSK3β (Ser9), GSK3β, p-β-catenin (Ser33/37/The41) and non-p-β-catenin (Ser33/37/Thr41) were purchased from Cell Signaling Technology (Danvers, MA), and those against β-catenin and c-myc were purchased from BD Transduction Laboratories (Lexington, KY) and Santa Cruz Biotechnology Inc., (Santa Cruz, CA), respectively. After incubation with HRP-conjugated secondary antibodies, images were visualized by enhanced chemiluminescence (Pierce). Signal intensity for p-AKT and total AKT was quantified by LAS3000 (Fujifilm, Tokyo, Japan).

2.7. Statistical analysis

All data are shown as mean ± SD. Statistical significance was determined by Mann–Whitney's *U*-test with the software JMP 9.0 (SAS Institute Japan, Tokyo, Japan). *p*-values less than 0.05 were considered significant.

3. Results

3.1. F344 rats are more susceptible to PhIP-induced colon tumorigenesis and obesity than ACI rats

Whereas carcinogenicity of chemicals is generally correlated with the number of ACF induced at an early point [16], this relationship remains elusive for PhIP. To address this issue, we chronologically monitored the colons from two rat strains treated with an intermittent PhIP-feeding protocol [11] (Fig. 1A). The numbers of both ACF and aberrant crypts (ACs) were significantly higher in the F344 rats compared to those in ACI rats at 12 weeks of age (Fig. 1B), consistent with a previous report [12]. At later time points, the number of ACs was still significantly higher in the F344 rats than in the ACI rats, but not with ACF. At 38 weeks of age, the number of colon tumors was significantly higher in F344 rats (Fig. 1C). In addition, the total number of dysplastic ACF, adenoma and adenocarcinoma were all higher in F344 rats. These results clearly indicated that the F344 rats more potently develop more advanced lesions than the ACI rats at any time point. Consequently, we reasoned that the number of ACF at 12 weeks of age would in fact serve as a marker to estimate tumor susceptibility of the strain and was used in subsequent analyses.

While examining ACF, we noted that the F344 rats tended to have more fat than the ACI rats. To verify this notion, we strictly quantified the fat weight of both strains at 6–12 weeks of age under LFD. Despite the similar level of body weight, a significantly higher degree of fat deposition was observed in F344 rats (Fig. 1D). This was also the case for visceral and subcutaneous fat. Severe accumulation of visceral fat has been associated with metabolic syndrome, which is characterized by hyperlipidemia, hypercholesterolemia and type II diabetes [17]. We then conducted

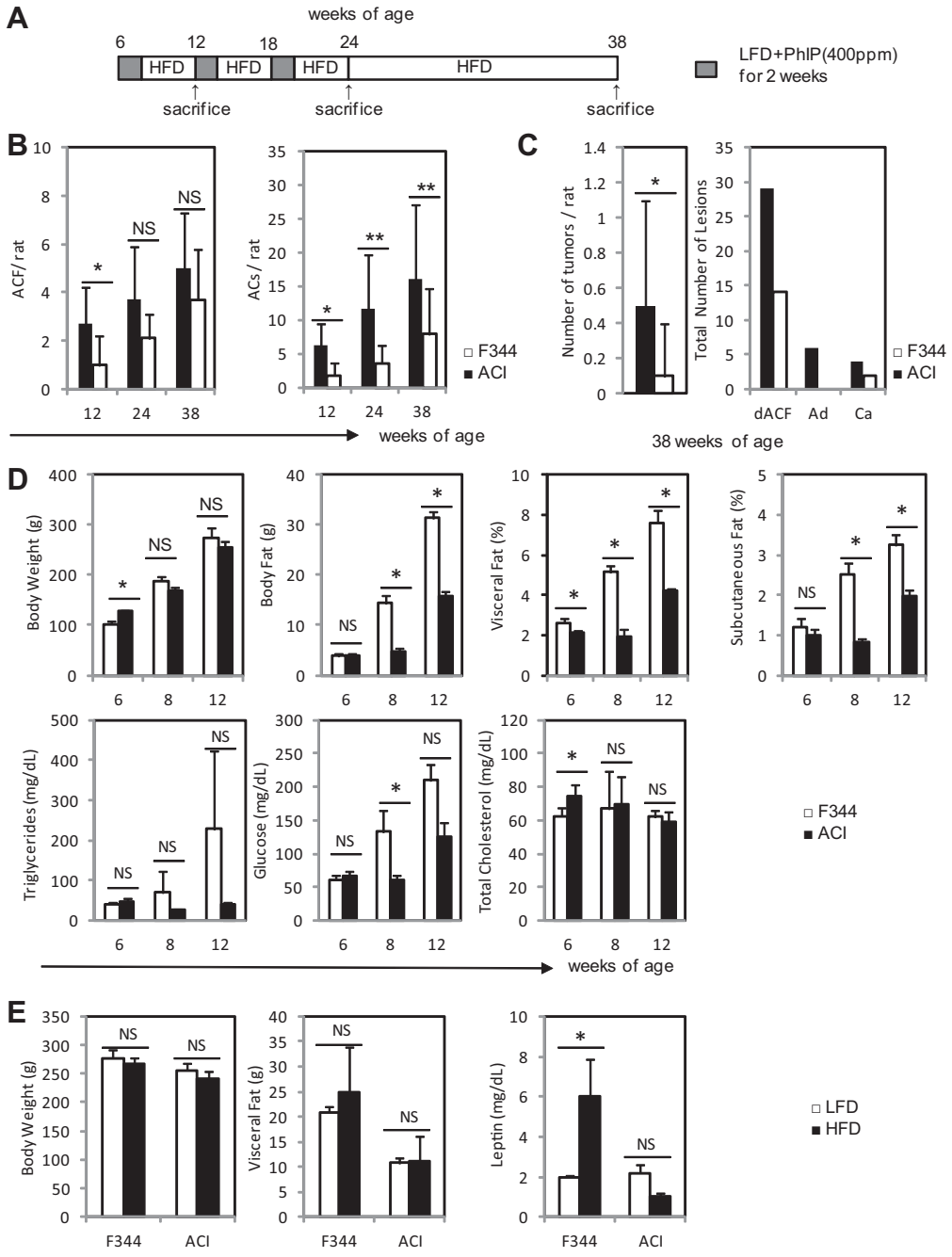

Fig. 1. Higher susceptibility to PhIP-induced colon tumorigenesis and obesity in F344 rats than in ACI rats. (A) A schematic view of the intermittent protocol for PhIP-induced colon carcinogenesis. (B) Time-series analysis of the number of ACF and ACs. The colons were examined at 12 (n = 10 each), 24 (n = 10 each), and 38 (n = 20 each) weeks of age. (C) Total number of tumors at 38 weeks of age. Both adenoma and carcinoma were counted as tumors. dACF, dysplastic ACF. Ad, adenoma. Ca, adenocarcinoma. (D) Time series analysis of body fat weight and blood biochemistry. Rats under LFD were sacrificed at 6 (n = 4 each), 8 (n = 4 each), and 12 (n = 3 each) weeks of age. (E) The effects of HFD on obesity. F344 (n = 4 each) and ACI (n = 5 each) rats under LFD or HFD for 6 weeks were sacrificed at 12 weeks of age. $^*p < 0.05$ NS, not significant.

a blood biochemistry test and found that the level of serum triglycerides (TG) and glucose, but not total cholesterol, tended to be higher in F344 (Fig. 1D). Given that PhIP-induced colon carcinogenesis is promoted by HFD, we examined the effects of 6-week HFD on obesity. During 6–12 weeks of age, neither body weight nor the amount of visceral fat was affected in either strain (Fig. 1E). By contrast, the level of serum leptin significantly increased in F344 rats under HFD (Fig. 1E), in line with increased fat intake and ruling out the possibility that rats were improperly fed. These results indicated that F344 rats are inherently more prone to both CRC and obesity than ACI rats, which could be evaluated by measuring ACF and TG at 12 weeks of age.

3.2. Correlation between the magnitude of obesity and the incidence of ACF across multiple strains

We wondered if the observed correlation between predisposition to obesity and CRC could be more generalized. In an effort to identify genetic determinants of susceptibility to PhIP-induced CRC, we had characterized a total of 18 independent rat strains in terms of incidence of ACF under HFD for 4 weeks and collected blood samples, albeit under non-fasting conditions, from rats under LFD for 4 weeks (Fig. 2A). Although these data and samples may not be ideal for accurate analysis, we took advantage of this situation to gain insights into the common predisposition. Plotting the incidence of ACF (Fig. 2B)

and serum lipid level for each strain revealed a correlation between incidence of ACF and TG, but not cholesterol (Fig. 2C). Out of the 18 strains, we selected six strains, readily available and with relatively strong correlations, for more detailed analyses under strict conditions. Specifically, BUF, LEW, F344, and LEA, ACI, NIG-III, were postulated to constitute a tumor- and obesity-prone subgroup and a resistant subgroup, respectively. Both body fat weight and body fat percentage, with the exception of LEW rats, were indeed high in a tumor- and obesity-prone subgroup (Fig. 2D). Similar results were obtained for TG levels in a fasting state, but not cholesterol or glucose levels (Fig. 2E). These results suggested that predisposition to obesity and PhIP-induced colon tumorigenesis in the five strains might be regulated by a common mechanism.

3.3. Correlation between the magnitude of AKT activation and incidence of ACF

To gain insights into the molecular basis for the common predisposition, we set out to determine genes differentially expressed in the colons between F344 and ACI, under LFD and without PhIP. We performed Gene Set Enrichment Analysis (GSEA), which revealed a number of differentially expressed pathways (Table S1). We focused on the PI3K/Akt pathway (Fig. 3A) on the list, because it has been already implicated in both carcinogenesis and metabolism [18]. As many upstream regulators and downstream effectors of the PI3K/Akt pathway are subject to regulation by phosphorylation, we examined the level of ~30 proteins in the pathway for total protein and phosphorylated protein by Western blot analysis. We eventually found that the magnitude of AKT activation had a good correlation with colon tumor susceptibility among the five selected rat strains (Fig. 3B). In line with this observation, FOXOs and Bim, pro-apoptotic molecules inhibited by AKT, were downregulated in the crypts from the strains with higher tumor susceptibility (Fig. 3C).

3.4. Activation of AKT in the colonic cells by PhIP in vivo and in vitro

Having confirmed the static link between the common predisposition and the level of activated AKT in a basal condition, we next investigated whether PhIP and/or HFD could dynamically regulate the magnitude of AKT activation in the colon. To achieve the highest sensitivity in detecting any alterations, we selected BUF rats, which manifested the most pronounced AKT activation (Fig. 3B). Western blotting revealed that AKT was hyper-activated exclusively in colonic crypts from the subgroup treated by PhIP for 2 weeks, followed by LFD for 4 weeks (Fig. 4A). In line with this

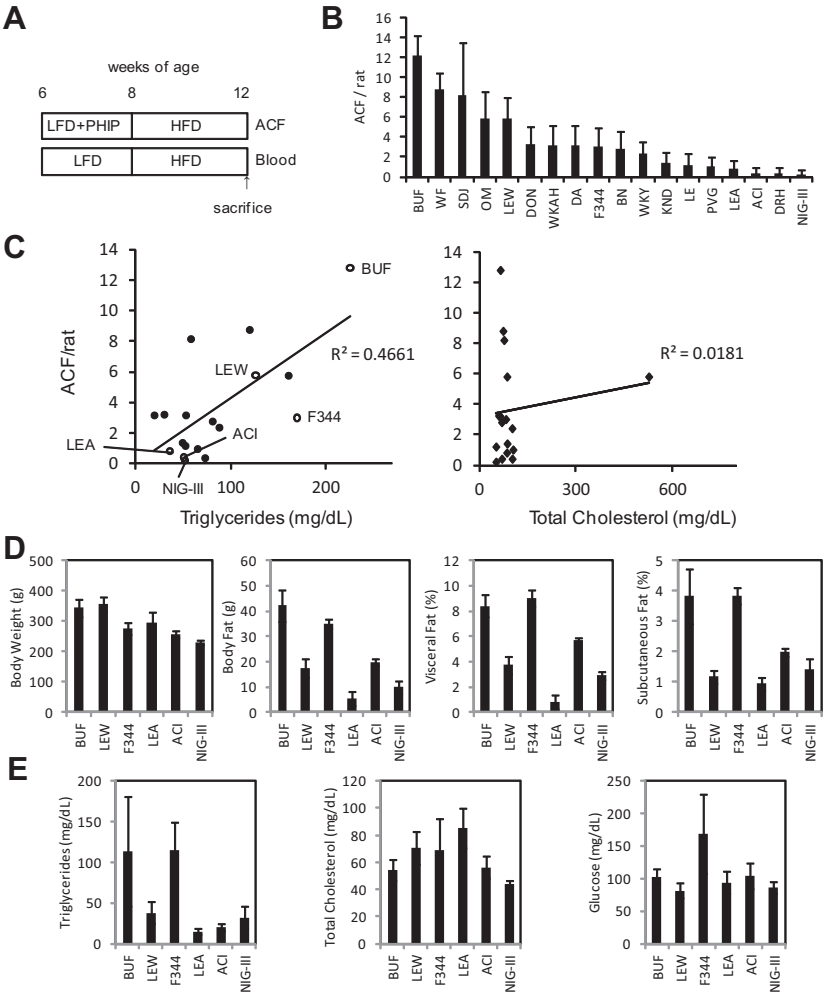

Fig. 2. Correlation between susceptibility to obesity and PhIP-induced colon tumorigenicity. (A) Feeding protocols. (B) The number of PhIP-induced ACF across 18 rat strains (n = 5 or 6 each). (C) Correlation between the number of ACF and amount of serum lipid. Triglyceride (*left*), and total cholesterol (*right*). Each circle depicts the mean level of serum lipid taken in a non-fasting condition from 5 individuals of each strain. Open circles labeled by strain name were used in the subsequent analysis D and E. Evaluation of obesity (D) and blood biochemistry (E) in six selected strains. Rats under LFD without PhIP for 2 weeks and subsequently under 4 weeks of HFD were examined (n = 5–11 each). BUF and F344, but not LEW, manifested an obesity phenotype, while ACI, LEA, and NIG-III did not (D). Only the level of serum triglyceride exhibited higher in BUF and F344, compared to the others (E).

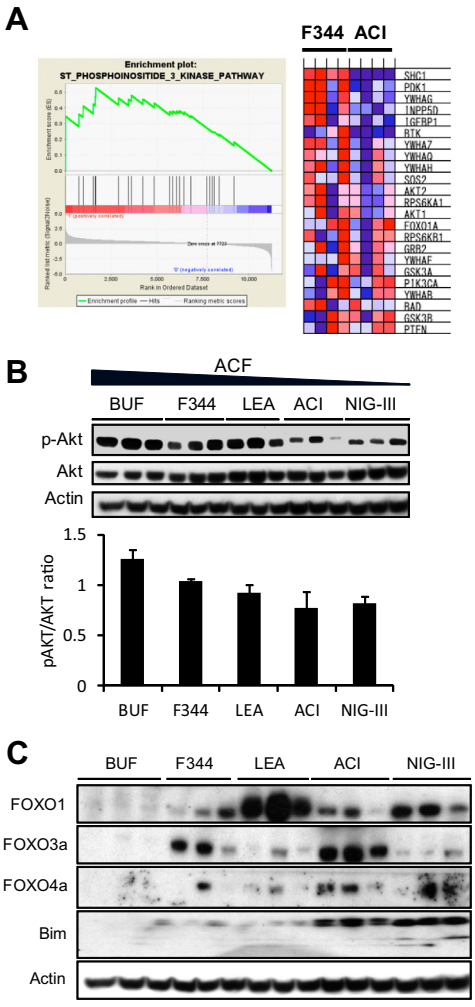

Fig. 3. Basal levels of pAKT in the colon correlate with predisposition to obesity and colon cancer. (A) Gene set enrichment analysis. The colonic crypts from F344 and ACI rats ($n = 4$ each) were subject to microarray analysis. Genes in the PI3K pathway overrepresented in differentially expressed genes (*left*). A heat map showing PI3K pathway genes (*right*). (B) Correlation between magnitude of AKT activation and the incidence of ACF. Western blotting analysis ($n = 3$) of the colonic crypts for total AKT, p-AKT (*upper panel*). Signal intensity ratio of p-AKT to total AKT correlated with the number of PhIP-induced ACF (*lower panel*). (C) Expression of pro-apoptotic molecules downstream of AKT. Western blotting analysis ($n = 3$) revealed FOXO family genes and Bim tended to show lower expression in tumor-prone rat strains. β-actin serves as a loading control.

observation, GSK-3βa key substrate of AKT, was phosphorylated specifically in the same group. The increase of pAKT was marginal without PhIP treatment or under HFD, even after PhIP treatment. To determine if the activation is achieved by a direct effect of PhIP on colonic cells, we treated human normal colon cells FHC with 10 μM of acetoxy-PhIP, a biologically active form of PhIP. Phosphorylation of AKT was indeed observed *in vitro*, albeit at a very early point and in a transient manner (Fig. 4B). These results implied that PhIP directly and promptly activates AKT, which could be sustained *in vivo* only under LFD, by an unknown mechanism.

3.5. PhIP and HFD inhibited apoptosis and activated Wnt pathway by distinct mechanisms

Given that GSK-3β promotes degradation of β-catenin, inactivation of GSK-3β by AKT is supposed to result in β-catenin accumulation leading to Wnt pathway activation. Indeed, PhIP-induced AKT activation increased the amount of total β-catenin, consistent with an earlier study [19], but to a lesser extent compared to HFD

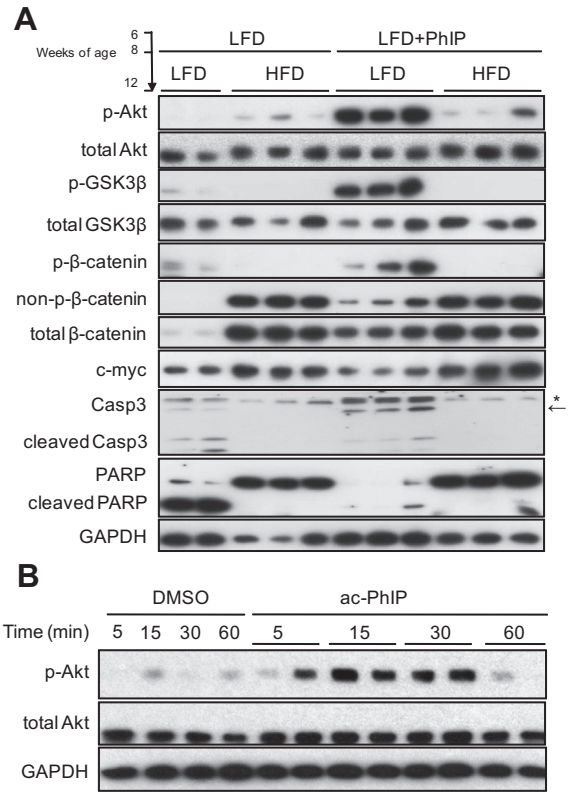

Fig. 4. Wnt pathway activation and inhibition of apoptosis by PhIP and HFD. (A) Characterization of key molecules in the Wnt pathway and apoptosis. Colonic crypts of the BUF rats from 4 subgroups ($n = 3$ each, $n = 2$ for LFD with PhIP) were analyzed by Western blotting analysis. GAPDH serves as a loading control. Non-specific bands (*asterisk*), specific bands (*arrow*) for full-length caspase3. Note that effects by PhIP were sustained even at 4 weeks later under LFD, but not under HFD. (B) Activation of AKT by PhIP in vitro. Normal human colon cells FHC were exposed to 10 μM N-acetoxy-PhIP (ac-PhIP). Negative controls were treated with DMSO ($n = 1$). Cells were collected at 5, 15, 30, and 60 min after PhIP treatment ($n = 2$).

(Fig. 4A). To qualitatively characterize β-catenin, we examined its phosphorylated and non-phosphorylated form, corresponding to an inactive and active form, respectively. It was revealed that HFD exclusively increased the amount of active β-catenin, while PhIP predominantly increased the amount of inactive β-catenin. In line with this observation, the level of c-myc, a major Wnt target gene, was indeed higher under HFD than upon PhIP treatment (Fig. 4A). AKT-induced elevation of inactive β-catenin, however, contradicts with the assumption that inactivation of GSK-3β should result in accumulation of active β-catenin, strongly suggesting that an alternative mechanism might be operating.

We next examined the effects of PhIP and HFD on apoptosis. Caspase3 and poly-ADP ribose polymerase (PARP) were predominantly in cleaved forms in colonic crypts under LFD, indicating massive apoptosis. By contrast, the cleaved forms were not detected under HFD or treated by PhIP, which seems to be achieved via distinct mechanisms. HFD suppressed expression of caspase3, thereby diminishing its cleaved form, while PhIP suppressed cleavage from full-length caspase3. Conversely, PhIP regulated PARP and caspase3 in a reciprocal manner (Fig. 4A). Collectively, HFD and PhIP activated the Wnt pathway and inhibited apoptosis, but through distinct mechanisms in the colon.

4. Discussion

Obesity, a major risk for CRC, has been generally implicated in progression from the initiation step of carcinogenesis. In the

present study, we showed that obesity could be also implicated in the early stages, by sharing a common genetic predisposition with PhIP-induced tumorigenesis. The common genetic predisposition appeared to be conveniently estimated by the level of serum TG and activated AKT in the colonic mucosa. AKT was also dynamically activated by PhIP, which seemed to be promoted by the intestinal microenvironment, but inhibited by HFD, underscoring the relevance of cooperation between genetic and environmental factors toward PhIP-induced colon carcinogenesis. Given the pro-survival properties of AKT and pro-tumorigenic effects of obesity, inhibition of AKT activation by HFD appears paradoxical in terms of tumor promotion. However, this observation might account for the reason why a cycling protocol alternating PhIP with HFD could induce colon tumors more efficiently than continuous exposure to PhIP [11].

PhIP-induced activation of AKT resulted in inactivation of GSK-3β as predicted, but did not lead to full activation of β-catenin for an unknown reason. Consequently, PhIP + LFD induced only a modest increase of the non-phosphorylated β-catenin compared to HFD. These results imply Wnt pathway-independent roles of GSK-3β inhibition in PhIP-induced tumorigenesis. In support of this notion, the colony formation potential of singly dissociated intestinal stem cells in 3D culture is significantly improved by a GSK-3β inhibitor, but not by Wnt3a ligands [20], raising the possibility that PhIP could promote survival of stem cells that might harbor mutations introduced by PhIP. Both PhIP and HFD inhibited apoptosis of colonic crypts, but surprisingly in a completely distinct manner that has never been reported previously. Expression of PARP was suppressed by PhIP, but induced and retained by HFD. As PARP is a component of the TCF4/β-catenin complex and positively regulates its transcriptional activity [21,22], its presence might contribute to a more pronounced activation of the Wnt pathway by HFD than by PhIP.

Considering high serum TG has recently emerged as a high risk factor for CRC in humans [23] [24], consistent with the present study, the findings from this study might have implications on personalized medicine. For instance, those individuals with high serum TG and AKT phosphorylation in the colon might constitute a subgroup with higher risk for CRC, even in the absence of macroscopic colonic lesions. Development of biomarkers for downstream of AKT would be also warranted, which would enable efficient reduction of cancer risk by patient education, early detection of cancer and therapeutic intervention. Taken together, we demonstrated the relevance of AKT in the development of PhIP-induced and obesity-related CRC, providing not only mechanistic insights, but also clinical implications on the diagnosis and prevention of CRC.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.12.059.

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